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Cellular and molecular mechanisms of sulfur mustard toxicity on spermatozoa and male fertility

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Sulfur mustard (SM) is a toxic compound that can target human spermatozoa. It induces a wide variety of pathologic effects in human reproductive organ, including disturbance in sexual hormones, testicular atrophy, impaired spermatogenesis, poor quality of sperm, defetcs in embryo development, childhood physical abnormalities, and severe fertility problems. However, molecular and cellular mechanisms of SM action on male reproductive health and human sperm function are not clear. Excessive production of reactive oxygen species (ROS) and resulted oxidative stress (OS) is likely a significant mechanism of SM action which can be associated with sperm DNA damage, membrane lipid peroxidation, reduced membrane fluidity, mitochondrial deficiency, apoptosis, and poor sperm quality. In this review, we aim to discuss cellular and molecular mechanisms of SM action on sperm and reproductive health, the significance of OS and mechanisms by which SM enhances infertility rate among SM-exposed individuals.

Key words: Sulfur mustard, human infertility, sperm, oxidative stress, reproductive system

Introduction

Sulfur mustard (SM), is a lipophilic compound which has been applied as a chemical warfare agent. During Iran-Iraq war (1980-1988), the unconventional use of SM injured more than 100,000 Iranians in which one-third of them are still suffering from the chronic effects [1, 2]. A great number of studies have reported different pathological and clinical effects of SM exposure in various organs [3]. Although the eyes, skin and airway system are the primary targets of SM toxicity [4-6], immunological, hematological and neuropsychiatric abnormalities, gastrointestinal problems, and sleep disorders are the other main pathological findings [1, 7-10].

The reproductive organ is another significant target for SM toxicity. However, there are still conflict reports regarding the effect of SM on human sperm and male infertility. Previous studies reported that the infertility rate in SM-exposed men is ranged from 2.5% to 35% [11-13]. Disturbance in sexual hormones, structural damages such as testicular atrophy, impaired spermatogenesis, and poor quality of sperm are the proposed reasons by which SM affects human reproductive health and fertility outcome [14]. Nevertheless, the actual mechanism in which SM triggers these abnormalities is not-well considered.

Excessive production of reactive oxidative species (ROS) and oxidative stress (OS) seem to be a significant mechanism of SM action on human reproductive function [14]. Recent studies have indicated that SM accelerates OS through the massive generation of ROS from endogenous sources or decrease in antioxidant capabilities and oxidative DNA repair [15]. The resulted OS then, in turn, can damage DNA leading to chromosome instability, altered gene expression, apoptosis and cell death [16, 17]. SM can also form adducts with DNA, lipids and proteins [18], and suppress nucleic acid and protein biosynthesis, which is associated with ATP depletion and disruption of intracellular energy metabolisms. Therefore, SM toxicity can be resulted from the direct damage induced by alkylating cellular components or ROS overproduction and oxidative stress.

Since human sperm membrane contains higher percentage of unsaturated fatty acid in contrast with other cells, it is particularly susceptible to OS and ROS. Therefore, spermatozoa can be considered as a major candidate for the pathologic and cytotoxic effects of SM [19]. In the following sections, we will discuss the general reproductive effects of SM, as well as the significance of OS and the mechanisms by which SM induces ROS generation and antioxidants depletion in reproductive organs.

Gonadotoxicity effect of SM

Although several studies considered negative effects of SM on human reproductive health, data addressing the adverse effects of SM on sperm function and male infertility are increasing. A growing number of clinical investigations and experimental

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studies have revealed that SM affects male reproductive system through the several mechanisms, including disturbance of sexual hormones, testicular atrophy, sexual dysfunction, genital lesions, impaired spermatogenesis, and poor sperm quality [14] (Figure 1). Table 1 shows a list of human and animal based studies that considered chronic and severe effects of SM on male reproductive function and sperm quality.

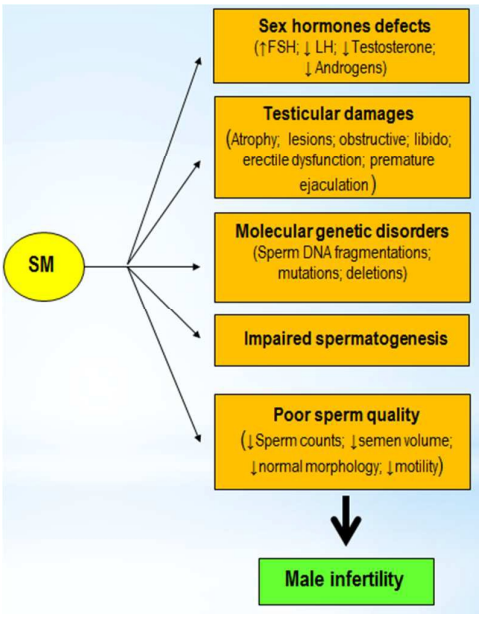

Figure 1. SM affects male reproductive system through the several mechanisms, including disturbance of sexual hormones, testicular damages, sperm DNA damages, impaired spermatogenesis, and poor sperm quality.

Table 1. Gonadotoxicity effects of SM on male reproductive function

Study model	Times after exposure	Findings	Refs
Human	Several years	↓fertility rate (23.3%); ↓quality of sperm (38.7%); ↑abortion (13.6%); ↑sexual dysfunction (9%); ↓libido (30%); ↑premature ejaculation (23.6%); ↑sex hormone deficiency, ↑FSH (57.6%); ↑LH (66.3%)	24, 25
Human	1st week	↓free serum testosterone (FT); ↓dehydroepiandrosterone (DHES)	89, 90
Human	5th week	↓FT; ↓DHES	20
Human	3rd and 5th week	↑serum FSH; ↑serum LH	20
Human	3 years	↓FT; ↑testicular atrophy; ↑impaired spermatogenesis; ↑Sertoli cells only pattern	20, 23, 91
Human	20 years	Normal LH, FSH and Testosterone	21
Human	3 months	↑Oligozoospermia (33.3%)	20
Human	4 years	↑total sperm counts	21
Human	10 years	↑abnormal sperm (38%); ↑sperm with abnormal morphology (54%); ↓sperm motility (48%)	13
Human	15 years	↑Oligozoospermia (10%)	11
Human	20 years	↓semen volume; ↓sperm counts; ↓sperm motility; ↓ sperm with normal morphology	21-23
Human	20 years	↑sperm with DNA damages	81
Human	8 years	↓libido (33.3%) ↑erectile dysfunction (9%); ↑premature ejaculation (23.6%)	25
Human	Few hours or few days	↑genital lesions; ↑hypopigmentation	2, 39
Male rats	10 days	↑abnormal sperm; ↓sperm counts; ↓sperm motility	29
Male rats	10 days	↑abnormal sperm; ↓Sperm counts; ↓sperm motility; ↓FT; ↓testicular weight	92

Structural changes and impaired spermatogenesis

Several lines of studies have shown that SM has a significant effect on testes structure and function. Testicular biopsy of SM-exposed patients revealed complete or relative arrest of spermatogenesis, atrophy of the germinal epithelium, but normal Sertoli and Leydig cells [20-23]. These data suggest that spermatogenesis is a significant target of SM toxicity. Spermatogenesis deficiency in SM-exposed individuals can provide further pathologic effects such as low semen volume because of ejaculatory duct obstruction, as well as poor sperm quality. Sexual dysfunction is reported among SM victims. In a study by Pour-Jafari

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et al., [24] among 800 SM-exposed Iranian men, 35% had decreased libido [24]. A previous study reported erectile dysfunction (9%) and premature ejaculation (23.3%) in SM-exposed patients [25]. These complications may be because of decreased level of serum testosterone. Other studies reported genital lesions such as hyperpigmentation, xerosis, and scars at the sites SM-induced injuries [26-28].

Effects of SM exposure on testes structure and spermatogenesis have been also studied in animal models. For instance, increased percentage of abnormal spermatozoa and impaired spermatogenesis were observed in male rats exposed to 0.50 mg/kg^{-1} SM [29]. Change in testicular integrity and decrease in testicular weight were detected in male rates after intraperitoneal injection of SM [30, 31]. Other studies reported that intravenous injection of SM in male mice caused testes damage and spermatogenesis deficiency [30, 32]. Furthermore, increased distance between seminiferous tubules, presence of necrotic forms of spermatocytes, and necrotic cells in the lumen were found eight weeks after SM-exposed rats [32]. Therefore, degenerative changes in testicular structure can be considered as one of the main mechanisms of SM that may be associated with impaired spermatogenesis, decrease in the number of spermatozoa, poor sperm quality and eventually male infertility.

Sperm quality

Several lines of studies indicated that SM exposure results in poor sperm quality, which suggests spermatozoa are particularly susceptible to cytotoxic effects of SM. For example, a previous study found azoospermia and severe oligospermia in 42.5% and 57.5% of SM- exposed patients, respectively [23]. Shakeri *et al*., [33] considered abnormal sperm morphology (53.8%), reduced sperm motility (48.4%), low sperm count (23.1%), as well as abnormal semen viscosity (17.6%) and declined semen volume (16.5%) in SM-exposed patients. In another study, semen analysis was performed for patients who exposed to SM during the Iran-Iraq war. The results showed sperm abnormalities in 38% of the SM victims [13]. In another research, the long-term effects of SM on the testis and male fertility were considered two decades after exposure. Male factor infertility was detected in 23% of SM-exposed patients and all semen indices were significantly decreased [21]. Therefore, these data suggest that spermatozoa can be a possible target for SM effects in the testis.

Deficiency of sexual hormone

SM exposure may disturb reproductive hormones, which are critical for regulation and initiation of spermatogenesis [34]. Furthermore, SM can interfere with the hypothalamus-hypophysis-testis axis, which is associated with impaired spermatogenesis and poor quality of sperm (Figure 2).

Gonadotropins, including follicle-stimulating hormone (FSH) and Luteinizing hormone (LH), and testosterone, are key regulators of germ cell development and spermatogenesis. Altered expression and secretion of gonadotropins and testosterone can be associated with abnormal spermatogenesis and male infertility. Previous studies showed significant changes in plasma levels of gonadotropins and testosterone in SM-exposed patients [20, 21, 23, 32, 35]. For instance, increased level of FSH was observed in serum of patients who exposed to SM [20, 21]. In a long-term study, Azizi *et al*., [20] found that exposure to SM reduces androgen level and hypo-responsiveness to GnRH. They also found that serum total and free testosterone and dehydroepiandrosterone were markedly decreased after SM exposure [20]. In another study, Agin and Sarvghadi [36] found reduced serum free testosterone levels in 32.6% of SM-exposed patients.

Since sperm counts are positively correlated with testosterone level, a marked reduction of intratesticular testosterone contents can initiate germ cell apoptosis in the seminiferous epithelium [37]. Therefore, any reduction of testosterone concentration caused by SM may interfere with the initiation of spermatogenesis, and lead to germ cell apoptosis and low quality of sperm. Additionally, there is a significant relationship between high serum FSH level with reduced number of sperm and abnormal morphology of spermatozoa [20]. Increased FSH level is an indicative of abnormal spermatogenesis and may suggest primary testicular failure. These findings indicate that a reduced sperm count in SM exposed patients is attributable to a primary testicular injury and a proof supporting the idea of SM gonadotoxicity [21]. However, it seems that serum levels of the reproductive hormones are within the normal range in SM-exposed men several years after the injury, which is dose depended [14].

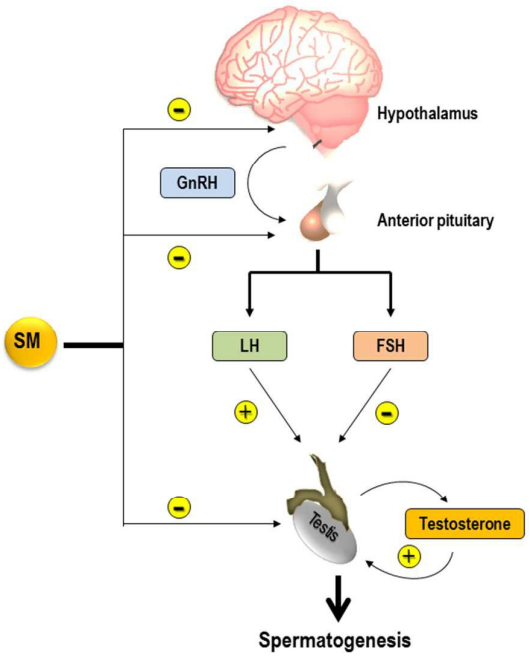

Figure 2. The pathologic effects of SM on hypothalamus-hypophysis-testis axis which disrupt the reproductive hormones and spermatogenesis process

Mechanisms of SM action

Since SM is a lipophilic compound, it can be easily absorbed and quickly entered into the body through the eyes, skin and respiratory system [26]. Afterward, it distributes systemically through the circulatory system and affects various organs, especially reproductive system. Recent evidences have suggested that SM toxicity is mediated through the several mechanisms such as damages to macromolecules, depletion of cellular nicotinamide

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adenine dinucleotide (NAD), increase of cellular calcium levels, increase of apoptosis mediators, oxidative stress, inflammation, and cellular antioxidant depletion [38] (Figure 3).

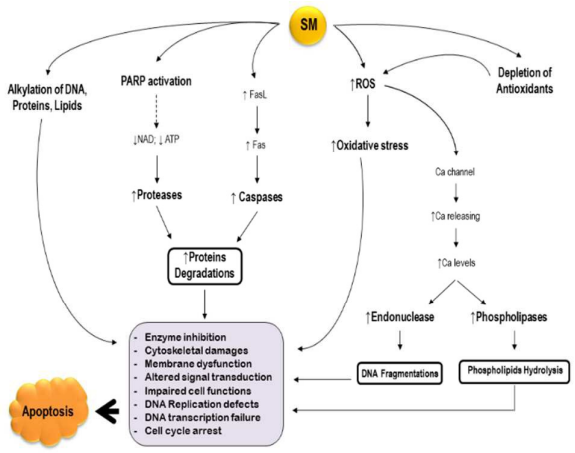

Figure 3. Possible cellular and molecular mechanisms of SM action on apoptosis and cells death

Damages to macromolecules

When SM absorbed, it undergoes intramolecular cyclization and forms a sulphonium ion, which in turn alkylates DNA, lipids and proteins, leading to DNA strand breaks and consequently cell death [39, 40]. These cellular effects are associated with tissue responses such as synthesis and secretion of inflammatory mediators and tissue damage (Figure 3) [41].

DNA damage is the primary initiator of the cellular responses which is associated with clinical injuries [8]. SM induces different structural modifications in DNA, which can lead to DNA strands breaks, genotoxic stresses, proteins or genome modifications, deficiency of DNA replication and transcription, cell cycle arrest, apoptosis and cell death [18]. SM can also directly interact with proteins and interfere with their normal function through the miss folding, oxidation, cross-linking and enzyme disability [38]. Lipids are the other targets for SM that can be peroxidized when exposed to SM, and then free radicals will be released as byproducts of lipid peroxidation [14].

Depletion of nicotinamide adenine dinucleotide

NAD depletion is another mechanism of SM action. Upon SM-induced DNA damage, DNA repair systems such as Poly (ADP-ribose) polymerase (PARP) pathway, base excision repair, and nucleotide excision repair are activated [38]. Recent evidences have revealed that DNA breaks induce PARP activation that lead to NAD^+ or ATP depletion and stimulation of the $NADP^+$ dependent hexosemonophosphate shunt; this event in turn enhances synthesis and release of proteases [42]. Increased expression and activation of proteases is associated with cell death and tissue injuries [43] (Figure 3). Previous studies demonstrated that the PARP produces poly-(ADP-ribose) (PAR) alone that induces signals for apoptosis and cell death [44].

Calcium (Ca^{2+}) releasing

Recent evidences have considered calmodulin and increases in intracellular Ca^{2+} levels as a signaling molecule induced by SM exposure [45]. Calmodulin and increased content of intracellular Ca^{2+} play an important role in apoptosis and cell death (Figure 3). Cytosolic calcium can be increased by the activity of protein kinase (PK) signaling pathways that lead to the activation of phospholipase C (PLC) and the generation of inositol triphosphate (IP3), which acts on calcium channels to release it from intracellular stores [46]. Another possible mechanism of cytosolic Ca^{2+} enhancement is due to the massive production of reactive oxygen species (ROS) caused by SM. ROS react with Ca^{2+} transport channels inside the endoplasmic reticulum, mitochondria, and cell membrane. These interactions damage the Ca^{2+} transport channels, which result in an influx of Ca^{2+} into the cytosol [47]. Increased contents of cytosolic Ca^{2+} not only induce activity of proteases such as Caspases, but also it induces Phospholipases and Endonucleases activity which in turn degrade cellular proteins, lipids and DNA [48] (Figure 3).

Mediators of apoptosis

Previous studies demonstrated that SM induces the overexpression of FasL and Fas, as an apoptotic signaling, in damaged cells [49]. FasL and Fas induce Caspases activation, which in turn lead to protein degradations and apoptosis (Figure 3). The other signaling molecules such as NF-κB, p38, and p53 are mediator factors that trigger numerous cellular responses such as inflammation, apoptosis, proliferation, and differentiation [50, 51]. SM induces these mediators and leads to inflammation, apoptosis or cell death in SM-damaged cells.

Oxidative stress and infertility of men

Massive generation of ROS and OS is likely a main reason for poor sperm quality and male infertility in SM exposed patients. Oxidative stress is defined as the imbalance between the ROS generation and the cellular antioxidant systems [52]. ROS are highly reactive free radicals which are produced by living organisms during normal cellular metabolism [52, 53]. At high concentrations, they can interact with lipids, proteins, and DNA and adversely affect certain cellular processes and modify normal cells function [54]. However, ROS are critical for normal sperm function such as acrosome reaction and sperm capacitation at low concentrations [55].

OS has been suggested as one of the main reasons for low quality of sperm and male infertility [55-57]. Recent studies have indicated that immature spermatozoa or abnormal sperm cells and leukocytes are the major sources of ROS in human semen [58]. ROS target sperm membrane lipids, DNA and proteins; alter enzymatic systems; produce irreversible alterations; cause cell death; and ultimately, lead to a decline in the semen parameters associated with male infertility [58] (Figure 4).

ROS can decrease the fluidity of sperm plasma membrane, leading to loss of the sperm ability for oocyte fusion and fertilization [59]. Since human spermatozoa contain high percentage of polyunsaturated fatty acids (PUFA) in their plasma membrane, they are very susceptible to ROS [60]. PUFA are critical for the fluidity of sperm membrane, ion transport, and sperm capacitation within the female reproductive tract. Therefore, sperm lipid peroxidation negatively affects membrane function, its transport activity and eventually surviving of spermatozoa (Figure 4). Lipid peroxidation has also a deleterious effect on the ultramorphological structure of sperm cells and thereby on the

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male fertilization potential [61]. Oxidation of sperm membrane lipids axonemal proteins can be associated with permanent impairment of sperm motility because excessive ROS deplete cellular ATP resulting in decreased phosphorylation of axonemal proteins and cause transient impairment of motility, as well as decreased sperm viability [58].

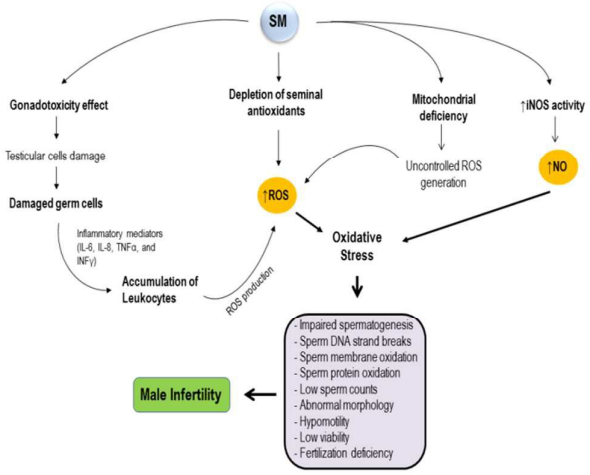

Figure 4. Mechanisms through which SM induces oxidative stress and male infertility

Numerous studies have also revealed that ROS can target sperm DNA by causing base modification, DNA strand breaks, DNA fragmentation and deletions, mutations, and chromatin cross-linking [61-65]. DNA damages can increase germ cells apoptosis and reduce sperm counts [66] (Figure 4).

In order to counteract the toxic effects of ROS, human seminal plasma and spermatozoa are equipped with enzymatic and non-enzymatic antioxidants that act as ROS scavenger, thereby protecting sperm cells from oxidative damage [58]. The seminal plasma antioxidants are very important because they compensate the depletion of sperm cytoplasmic enzymes when the cytoplasm is extruded during maturation [67]. Nevertheless, overproduction of ROS in reproductive organ can overwhelm the effective contents of antioxidants, increasing the harmful effects of ROS to spermatozoa that are associated with abnormal sperm parameters [68]. SM can lead to excessive production of ROS causing progressive oxidative damage and ultimately sperm cells death.

Role of SM in oxidative stress and inflammation

OS induced by free radicals is now believed as one of the main mechanisms of SM toxicity [69, 70]. SM increases ROS production and OS via several mechanisms, including accumulation of leukocytes and inflammation, reduced activity of antioxidants, enhanced expression of ROS producing-related enzymes, mitochondrial dysfunction, depletion of glutathione (GSH) and productivity of GSH-dependent antioxidant enzymes, as well as change in activity of inducible nitric oxide synthase (iNOS) [71] (Figure 4).

A growing number of studies have confirmed a close relationship between the presence of leukocytes in semen and male infertility [63]. Some studies have revealed that elevated levels of seminal ROS, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) are associated with increased content of sperm membrane lipid peroxidation and poor sperm quality [72-74]. Recent evidences have revealed that SM exposure is significantly associated with inflammatory reactions and oxidative injury at the site of damaged tissues [70, 75, 76]. Experimental studies showed that SM can induce the secretion of several proinflammatory cytokines and growth factors such as TNFα, IL-α, IL-β, IL-6, IL-8, IL-13, IL-15, and INF-γ in damaged tissues [77-80]. SM can also accumulate several inflammatory cells such as macrophages and neutrophils with a subsequent release of inflammatory mediators that can recruit and activate other leukocytes in reproductive system [14]. Activated leukocytes generate high levels of ROS which in turn overwhelm the antioxidant defense systems, leading to increased OS in seminal plasma. Overproduction of ROS by SM-activated leukocytes cause oxidative damage to sperm DNA, protein and membrane PUFA, which are associated with further inflammations, impaired spermatogenesis, apoptosis and poor quality of sperm [81] (Figure 4).

Several studies have shown that SM induces mitochondrial dysfunction, which may be associated with electron transport chain deficiency, massive production of ROS, DNA oxidation and depletion of intracellular antioxidants [69, 82]. Spermatozoa are rich in mitochondria because a constant supply of ATP is necessary for their motility. Increased number of abnormal or immature spermatozoa significantly enhances ROS generation, which in turn affects their mitochondrial function and subsequently, sperm motility [58, 83].

SM can also impair spermatogenesis and induce sperm DNA fragmentation. In a previous study, the relationship between SM exposure and sperm DNA fragmentation was considered two decades after SM exposure [81]. A significant increase in sperm DNA fragmentation index was found in SM patients, indicating the increased risk of congenital abnormalities and genetic defects in SM-exposed victims offspring created by assisted reproductive techniques (ART) technique [22, 81].

SM can also decline the effective concentration of antioxidants through the enhancing of ROS generation (Figure 4). Glutathione (GSH) is a primary target for SM because its level has been markedly reduced after SM exposure [80]. SM-GSH metabolites decrease cellular GSH and increase intracellular ROS, as well as OS markers including DNA, lipid and protein oxidations [80]. Recent investigators have demonstrated that GSH and N-acethylcysteine (as a GSH prodrug) treatments, reduce OS and toxicity induced by SM [84-86]. SM can also decrease the activity of other antioxidants such as thioredoxin reductase, catalases (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione-S-transferases (GST), which are critical in the control of cellular antioxidants balance [79, 87]. Reduced activity of these antioxidants can occur as a result of SM-induced alkylation or changes in expression of theses enzymes.

NADPH cytochrome p450 reductase, which has a critical role detoxification of different toxic metabolites, is another target for SM [88]. Several research have shown that SM not only inhibits the reduction of cytochrome C, but also it prevents the activity of NADPH cytochrome p450 reductase and stimulates ROS generation [88].

Conclusions

SM induces a wide variety of structural and functional disorders in reproductive system, including deficiency of

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reproductive hormones, testicular cells damages, sexual dysfunction, spermatogenesis deficiency, poor sperm quality, and reduced fertility. OS is a major mechanism of SM action on human reproductive health. SM induces DNA fragmentation, lipid and protein oxidation and as the result sperm apoptosis. It induces OS in reproductive system via several mechanisms, including accumulation of leukocytes and inflammatory mediators, mitochondrial deficiency, enhanced activity of ROS-producing enzymes, reduced activity of intracellular antioxidants, GSH depletion and decreased productivity of GSH-dependent antioxidants, and consequently imbalances between the production and detoxification of ROS in cells. Therefore, antioxidant therapy may be helpful to protect reproductive function against SM-induced damages.

Conflicts of interest

No competing interest was declared by any of the authors.

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